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MINOR COMPONENTS OF WHEAT GERM s-RIBONUCLEATES

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DEPARTMENT OF BIOCHEMISTRY

by

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UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "MINOR COMPONENTS OF WHEAT GERM s-RIBONUCLEATES", submitted by Louise Laura Nielsen Hudson in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

The s-ribonucleates prepared from wheat germ were analyzed both qualitatively and quantitatively for minor constituents after hydrolysis by (i) alkali, and (ii) snake venom phosphodiesterase.

The alkali-hydrolysates of the wheat germ s-ribonucleates were fractionated on DEAE cellulose columns. The fractions containing nucleosides and nucleoside diphosphates accounted for 2.6 mole per cent of the constituent nucleotides of the s-ribonucleates. The nucleosides and diphosphonucleosides derive from terminal groups and were quantitatively analyzed in order to assess the chain length of the s-ribonucleates. The fraction containing alkali-stable dinucleotides also accounted for 2.6 mole per cent of the constituent nucleotides of the s-ribonucleates. There were fifteen alkali-stable dinucleotide sequences in the s-ribonucleates and their proportions were compared with the proportions of the corresponding sequences isolated from wheat germ 18S + 28S ribonucleates.

The products formed by hydrolysis of the s-ribonucleates with purified snake venom phosphodiesterase were separated by two-dimensional paper chromatography. The major nucleoside 5'-monophosphates accounted for 91 mole per cent of the total nucleotides present in the s-ribonucleates. Pseudouridine 5'-monophosphate accounted for 2.8 mole per cent of the constituent nucleotides of the s-ribonucleates while the

nucleoside 5'-monophosphates which have methyl substituents in the heterocycle (pN^{x}) accounted for 5 mole per cent of the constituent nucleotides of the s-ribonucleates. The products formed by the hydrolysis of the s-ribonucleates with whole snake venom were fractionated on DEAE cellulose columns and the fraction containing the 2'-0-methyl nucleoside 5'-monophosphates accounted for 1.3 mole per cent of the constituent nucleotides of the s-ribonucleates.

ACKNOWLEDGEMENTS

The author is indebted to Dr. B. G. Lane, under whose direction these studies were performed. Mr. M. Gray deserves special thanks for his studies of the nucleotides bearing methyl substituents on the heterocycle.

The author also wishes to thank Dr. C. M. Kay for the sedimentation-diffusion analysis of the s-ribonucleates.

The assistance of Mrs. L. Randall in the typing of the manuscript is gratefully acknowledged.

TABLE OF CONTENTS

LIST OF TABLES

Table Page*

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[*] The tables precede the pages indicated.

LIST OF ILLUSTRATIONS

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*The illustrations precede the pages indicated.

ABBREVIATIONS

s-ribonucleates - soluble ribonucleates

s.u. - spectrophotometric units (optical density at 260 mμ x volume)

methyl Cellosolve - 2-methoxyethanol

$E^{1\%}_{260}$ - the absorbance at 260 mμ of a 1% aqueous solution of ribonucleates

DEAE - diethylaminoethyl cellulose

n - bases (a, g, c, u)

N - nucleosides (A, G, C, U)

Np - nucleoside 2' or 3'-monophosphates

pN - nucleoside 5'-monophosphates

N^x - nucleosides with methyl substituents in the heterocycle

Nx - 2'-0-methyl nucleosides

pNp - nucleoside 2',5' (and/or) 3',5' diphosphates

NxpNp - alkali-stable dinucleotides

TRIS-formate - tris (hydroxy-methyl) aminomethane-formate

ψ - pseudouridine

I. INTRODUCTION

As recently as 1955, it was generally believed that the 2'- and 3'-nucleotides were the sole products of the alkali hydrolysis of ribonucleates (Brown and Todd, 1955). However, there have been recurrent reports that as much as 5 per cent of the ultraviolet absorbing material in the alkali hydrolysates of ribonucleates from many sources is non-mononucleotide in character.

Smith and Allen (1953) used two-dimensional paper chromatography to separate the components of neutralized alkali hydrolysates of yeast ribonucleates. In addition to the mononucleotides, there were compounds which had the properties of oligonucleotides. These compounds gave a negative test for deoxyribose and yielded the four bases adenine, guanine, cytosine and uracil when treated with strong acid.

Non-mononucleotide components in neutralized alkali hydrolysates of animal, plant and microbial ribonucleates were isolated by Smith and Dunn (1959a) using a combination of paper chromatographic and electrophoretic procedures. The non-mononucleotide components were shown to be dinucleotides. One of the nucleoside constituents of each dinucleotide possessed an unusual sugar with paper chromatographic properties similar to 2'- or 3'-O-methyl ribose. Biswas and Myers (1960) isolated an unusual pyrimidine derivative from acid hydrolysates of <u>Anacystis nidulans</u> ribonucleates. This was identified as a 2'- or 3'-O-methyl ribonucleotide by comparison of the constituent sugar with 2'- or 3'-O-methyl ribose.

A requirement for base catalyzed hydrolysis of phosphate diesters is the presence of a hydroxyl group cis to one of the phosphate ester linkages (Bailly and Gaume, 1955). Brown and Todd (1955) showed that cyclic 2',3'-phosphodiester nucleotide intermediates were formed in the acid and base catalyzed hydrolysis of benzyl and methyl esters of 2'- and 3'-nucleotides. Alkali and acid hydrolysis of ribonucleates also proceeds via the intermediate formation of nucleotide 2',3'-cyclic phosphates (Markham and Smith, 1952). Substitution by an O-methyl group at carbon 2' of a ribose residue in a ribonucleate chain would block the formation of a 2',3'-cyclic phosphate and therefore the phosphodiester linkage at the 3' position of the 2'-O-methyl sugar would be stabilized against alkali hydrolysis. Consequently a 2'-O-methyl riboside flanked by normal ribosides would be expected to appear as part of a stable dinucleotide after alkali hydrolysis of ribonucleates in accordance with the findings of Smith and Dunn (1959a).

Hall (1963, 1964) hydrolyzed ribonucleates from several sources by simultaneous treatment with snake venom, and alkaline phosphomonoesterase. Using partition chromatography on celite columns, the O-methyl nucleosides were isolated and shown to be 2'-O-methyl nucleosides and not 3'-O-methyl nucleosides. Morisawa and Chargaff (1963) isolated 2'-O-methyl guanosine and 2'-O-methyl cytidine from yeast s-ribonucleates and rat liver ribonucleates. They suggested that 2'-O-methyl adenosine and 2'-O-methyl uridine were absent from the ribonucleates examined.

Honjo _et al_. (1964) have recently characterized the

structure of the four 2'-0-methyl nucleosides from yeast ribonucleates by the classical techniques of organic chemistry.

Singh and Lane (1964a, 1964b) separated and identified alkali-stable dinucleotides from alkali hydrolysates of commercial yeast ribonucleates and 18S + 28S wheat germ ribonucleates. The existence of the 16 possible dinucleotide sequences involving the four principal nucleosides and 2'-0-methyl nucleosides was demonstrated in both of these ribonucleate preparations.

The available data on the quantitative proportions of the alkali-stable dinucleotide sequences in 18S + 28S ribonucleates from wheat germ, suggested that a comparison with s-ribonucleates from the same source might be informative. From the results of an investigation of s-ribonucleates, significant quantitative and qualitative differences have been shown to exist between the proportions of the alkali-stable dinucleotide sequences isolated from the 18S + 28S ribonucleates and the corresponding proportions found for the s-ribonucleates. A description of the work with alkali-stable dinucleotides from wheat germ s-ribonucleates will be the principal topic of this dissertation.

II. PREPARATION OF WHEAT GERM s-RIBONUCLEATES

The total ribonucleates were extracted from wheat germ in an aqueous phenol emulsion as proposed by Colter and Brown (1956) and by Kirby (1956). Freshly milled wheat germ of the Thatcher variety (125 g) was shaken for 20 minutes at room temperature in an emulsion composed of 500 ml of 0.05 M phosphate buffer, pH 6.8, and 500 ml of water-saturated phenol. The suspension was centrifuged for 15 minutes at 2,000 r.p.m. in an International Centrifuge refrigerated to 2°. In a typical preparation, aqueous layers from 17 extractions (17 x 125 g = 2125 g wheat germ) were pooled and made 3 M with respect to sodium chloride (Lane and Allen, 1961a). After a period of 18 hours at 5°, the viscous suspension was centrifuged to separate the insoluble ribonucleates. The supernatant solution was carefully removed from beneath a layer of phenol crystals, and after recentrifugation, the crude s-ribonucleates were precipitated from the supernatant solution by the addition of two volumes of 95 per cent ethanol (Lane and Allen, 1961a).

The suspension was allowed to precipitate for 18 hours at 5° before the bulk of the supernatant solution was decanted. The s-ribonucleates collected by centrifugation were purified by the method of Glitz and Dekker (1963). The combined pellets containing 67,800 s.u. of s-ribonucleates were dissolved in 750 ml of water. One volume of 2.5 M K_2HPO_4 (750 ml) and 0.05 volumes of 33 per cent H_3PO_4 (37.5 ml) were mixed together and added to the

opalescent solution of s-ribonucleates. Methyl Cellosolve (750 ml) was added to the phosphate buffered solution of crude s-ribonucleates and the resulting emulsion was shaken vigorously in a separatory funnel. The emulsion was then allowed to separate under gravity for 10 minutes. The lower phase was re-extracted with 150 ml of the upper phase of an emulsion made by shaking equal volumes of water, 2.5 M K_2HPO_4, methyl Cellosolve, and 0.05 volumes of 33 per cent H_3PO_4 in a separatory funnel. The combined upper layers from the s-ribonucleate extractions were dialyzed against cold running tap water for 18 hours.

The dialysate was made 0.1 M with respect to TRIS-chloride buffer, pH 7.6, and applied to a 35 x 4.5 cm column of DEAE-cellulose in the formate form. The column was washed with 3 liters of 0.1 M TRIS-chloride buffer, pH 7.6, and the s-ribonucleates were eluted using a linear concentration gradient of sodium chloride. The gradient of sodium chloride concentration increased linearly from zero to a final concentration of 1.0 M and the eluent was buffered throughout with 0.1 M TRIS-chloride, pH 7.6. Elution of the s-ribonucleates began after the passage of 1,000 ml of the gradient eluent into the column, when the sodium chloride concentration was approximately 0.5 M. The elution was followed by measuring the ultraviolet absorption at 260 mμ. Fractions with optical densities greater than 2 were pooled, made 1 M with respect to sodium chloride, and the purified s-ribonucleates were precipitated by adding 2 volumes of 95 per cent ethanol. After two days at 5°, the s-ribonucleates were collected by centrifugation,

TABLE I

The yield and purity of wheat germ s-ribonucleates after each stage of purification

Stage of purification	Yield (s.u.)	$E^{1\%}_{260\ m\mu}$
First ethanol precipitation	67,800	17
Methyl Cellosolve extraction	64,715	-
Dialysis	59,170	70
DEAE-cellulose chromatography	48,600	163*

*The purity of the product varied with different preparations but the $E^{1\%}_{260\ m\mu}$ never exceeded 187.

washed three times with 95 per cent ethanol, three times with ether, and air-dried. A summary of the yield and purity of the s-ribonucleates at different stages of purification is shown for this preparation in Table I.

TABLE II

The quantitative distribution of the products of alkali hydrolysis of wheat germ s-ribonucleates*

Type of compound	s.u.	% total s.u.
N	617	1.70
Np + $N^{x}p$	34,300	94.30
NxpNp	900	2.45
pNp	600	1.65

*1.7 g wheat germ s-ribonucleates was dissolved in 35 ml of water, made 1 M with respect to sodium hydroxide and hydrolyzed for 90 hours. The hydrolysate (36,400 s.u.) was fractionated on a 30 x 4.5 cm DEAE cellulose column in the formate form.

III. ANALYSIS OF ALKALI HYDROLYSATES OF WHEAT GERM s-RIBONUCLEATES

The data quoted in sections (i), (ii), (iv) and (v) of this part of the thesis specifically pertain to the preparation of s-ribonucleates which was described in detail in Part II of the thesis.

(i) Conditions for hydrolysis and the nature of the products

A 5 per cent aqueous solution of the s-ribonucleates was made 1 M with respect to alkali by the addition of 10 M sodium hydroxide solution, and was then allowed to stand at room temperature for 90 hours before neutralization with 1 M formic acid.

The types and relative amounts of the products formed by alkali hydrolysis of the s-ribonucleates are indicated in Table II. The principal products are the 2'- and 3'-isomers of adenylate, guanylate, cytidylate, uridylate and pseudouridylate (Np), together with smaller amounts of the 2'- and 3'-isomers of adenylate, guanylate, cytidylate and uridylate which have methyl substituents in the heterocycle (N^xp). Quantitative analysis of the Np compounds is complicated by the recent discovery of Röttger and Fritz (1962) which showed that the isomers of adenylate, cytidylate and uridylate are partially destroyed during alkali hydrolysis. Quantitative analysis of the N^xp compounds is complicated by the fact that there are only very small amounts of the individual compounds. Also the possibility that the 2'- and 3'-isomers of the N^xp compounds may resolve during paper or column chromatographic separations reduces the facility and precision with which they can be analyzed. These analytical drawbacks do not arise in

the analysis of the corresponding 5'-nucleotides (pN and pN^x) produced by phosphodiesterase hydrolysis of s-ribonucleates. Consequently hydrolysis by phosphodiesterase has been used to estimate pN and pN^x (cf. Part IV, (i)) and analyses of Np and N^xp in alkali hydrolysates were not performed. At least two of the N^xp compounds, 1-methyl adenylate and 7-methyl guanylate are completely destroyed during alkali hydrolysis (Lawley and Brooks, 1963) but the corresponding 5'-nucleotides are partially recoverable from phosphodiesterase hydrolysates.

The nucleosides (N), alkali-stable dinucleotides (NxpNp) and diphosphonucleosides (pNp) have been examined carefully and are not subject to significant destruction in alkali digests. It has been found that the nucleoside fraction from anion-exchange separations contains not only adenosine, guanosine, cytidine and uridine, but also the alkali breakdown product of 7-methyl guanylate and some adenine. The diphosphonucleoside fraction contains the 2',5'- and the 3',5'-diphosphates of adenosine, guanosine, cytidine and uridine. The total amount of nucleosides is equimolar with the total amount of diphosphonucleosides since they derive from opposite ends of the s-ribonucleate chains. The alkali-stable dinucleotide fraction is comprised of compounds which contain equimolar quantities of normal nucleoside and 2'-O-methyl nucleoside constituents.

(ii) Column fractionation of alkali hydrolysates of s-ribonucleates on DEAE cellulose

Neutralized alkali hydrolysates of s-ribonucleates were

diluted with water to reduce the salt concentration to 0.025 M. The diluted solution was applied to a DEAE cellulose column in the formate form which had been pre-equilibrated with 0.025 M TRIS-formate buffer, pH 7.8. The DEAE cellulose was packed under a pressure of 5 p.s.i. into columns which were either 2.5 cm or 4.5 cm in diameter and had a bed volume of 90.4 cc of DEAE cellulose for each 10,000 s.u. of nucleotide material in the charging solution. After the charging solution had passed through the column, elution was continued with 0.032 M TRIS formate buffer, pH 7.8, until the optical density (260 mμ) of the effluent was zero. The principal nucleate derivatives from the charging and washing solutions were the nucleosides from terminal groups together with small amounts of adenine and 2-amino, 6-hydroxy, 5-methyl formamido, 4-phosphoribosyl amino pyrimidine from the alkali degradation of 7-methyl guanylate.

Mononucleotides were eluted by an aqueous solution which was 0.085 M with respect to TRIS formate buffer, pH 7.8, and 7 M with respect to urea. The elution was continued until the optical density (at 260 mμ) decreased to that of the eluting buffer (approximately 0.030).

The alkali-stable dinucleotides were then eluted by an aqueous solution which was 0.170 M with respect to TRIS formate buffer, pH 7.8, and 7 M with respect to urea. The urea in the eluent made the elution pattern much sharper and eliminated the trailing of the purine nucleotides (Tomlinson and Tener, 1963). The elution was continued until there was a constant optical density (at 260 mμ) characteristic of the eluting buffer (approximately 0.030).

Diphosphonucleosides were eluted by a solution which was 0.25 M with respect to TRIS formate buffer, pH 7.8, and 7 M with respect to urea in the early stages of investigation. A more convenient method for the elution of nucleoside diphosphates, which was adopted for routine use in the later stages of the investigation, employed a volatile buffer to facilitate desalting of the effluent. The nucleoside diphosphates were eluted with 1 M pyridine formate, pH 4.5, after careful washing of the DEAE cellulose column with water to remove non-volatile TRIS formate and urea from the interstices of the column bed.

(iii) Paper chromatographic systems for separating nucleate derivatives

The following three systems have been used for paper chromatographic separations of the nucleate derivatives.

System 1. Whatman No. 1 filter paper was impregnated with ammonium sulfate by dipping the paper through a solution which was made by mixing 10 volumes of saturated aqueous ammonium sulfate solution and 90 volumes of water. The papers were air dried by suspension in a fume hood. Chromatograms were developed by the descending technique for a period of 18 hours using a solvent composed of 80 volumes of 95 per cent ethanol and 20 volumes of water. Although a development of 18 hours was used to separate most compounds, occasionally shorter periods of development were used. The chromatographic tank was equilibrated with the vapour of the developing solvent before

Ultraviolet photograph of a one-dimensional paper chromatographic separation of a variety of nucleate derivatives using System 1.

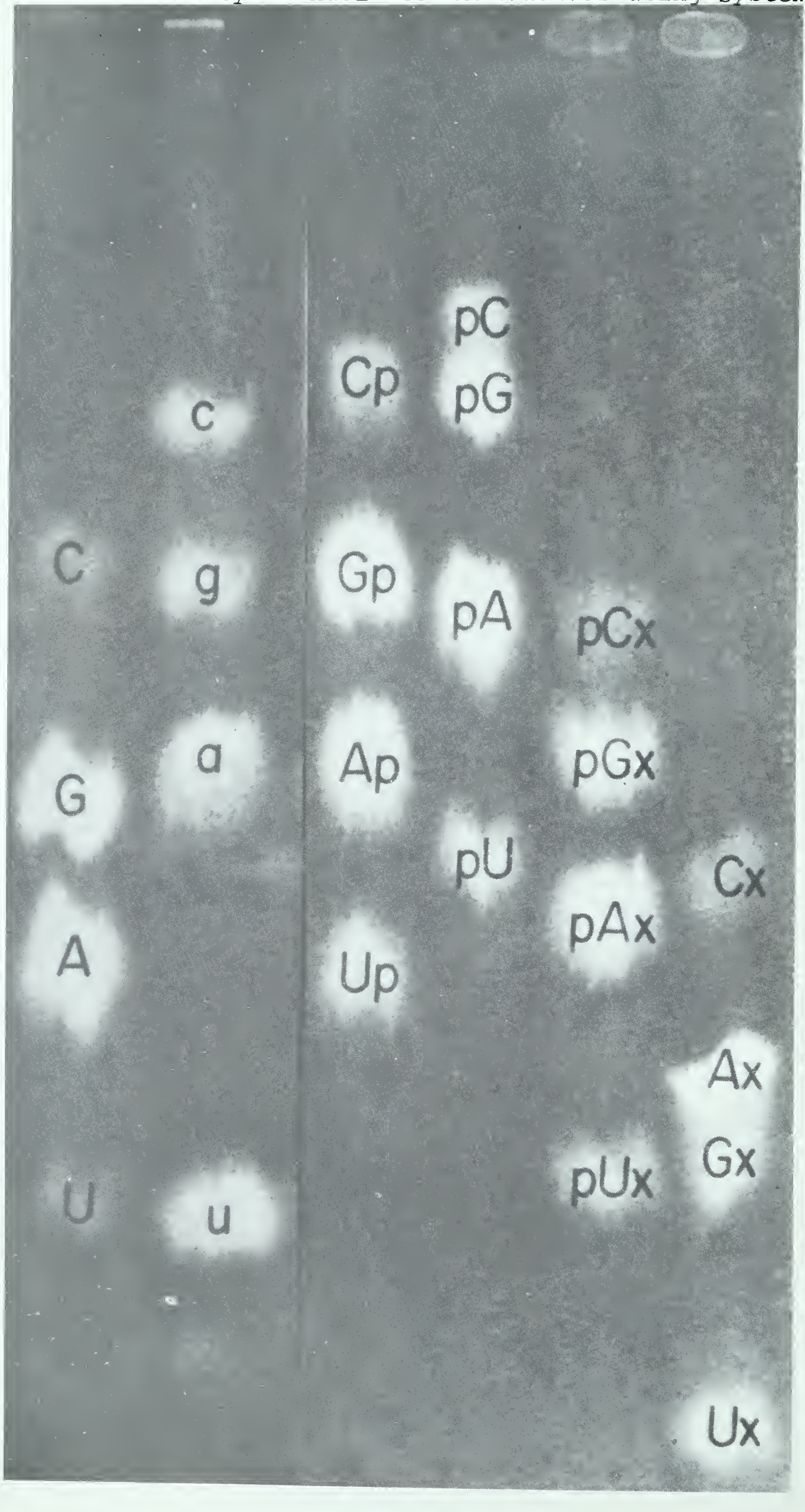


FIGURE 1

use but the papers were not pre-equilibrated with the vapour of the tank before development (Lane, 1963). An ultraviolet photograph of the chromatographic separation of nucleate derivatives in this solvent is shown in Figure 1.

System 2. The solvent is a slight modification of that introduced by Markham and Smith (1952). Whatman No. 1 filter paper impregnated with ammonium sulfate was used with a solvent prepared by the addition of 2 volumes of isopropanol to 80 volumes of saturated aqueous ammonium sulfate. Chromatograms were developed by the descending technique for a period of 15 to 18 hours.

System 3. In this system the developing solvent was composed of 80 volumes of 95 per cent ethanol and 20 volumes of 1 M ammonium acetate, pH 9.5, saturated with boric acid (Plesner, 1955). The chromatographic tank was pre-equilibrated by placing 2 beakers, each containing 100 ml of 75 per cent ethanol, on the floor of the tank. The filter papers were equilibrated with the vapour in the tank for one-half hour before development of the chromatograms for a period of 6 hours.

(iv) Quantitative analysis of the nucleosides and nucleoside diphosphates derived from the terminal groups of s-ribonucleates

The nucleoside fraction from column separations was desalted by charcoal adsorption and elution. An aliquot of the nucleoside fraction containing 15 s.u. was passed into a column 2.5 cm in diameter which consisted of a disc of charcoal (50 mg)

packed between two layers of celite. The column was washed carefully with 200 ml of water and the nucleosides were eluted with an eluent made by mixing 60 ml of water, 60 ml of 95 per cent ethanol, and 4 ml of concentrated ammonium hydroxide. The effluent was evaporated to dryness and the nucleosides were recovered by rinsing the flask with 2 ml and then again with 1 ml of water. The pooled washings (approximately 2.8 ml) were evaporated to dryness in a small evaporating tube and the residue was dissolved in 200 µl of water for chromatography in System 1 for 16 hours. The recovery of the nucleosides from column effluents was 85 to 90 per cent.

Four bands of nucleosides were recovered from the chromatogram and three of these, adenosine, cytidine and uridine appeared to be spectrophotometrically pure. The guanosine band had an unusual spectrum and so the nucleosides from 1.7 gm of s-ribonucleates were resolved by paper chromatography in System 1 and the material of the guanosine bands of several chromatograms was eluted, pooled and de-salted for further characterization. Paper chromatography using System 3, separated the material into 3 bands which were spectrally characterized, in order of increasing R_f value as guanosine; 2-amino, 6-hydroxy, 5-methyl formamido, 4-phosphoribosyl amino pyrimidine; and adenine. The pyrimidine derivative, a breakdown product of 7-methyl guanylate is neutral at pH 7.8 and thus appears in the nucleoside fraction. The adenine is probably a breakdown product of adenylate and only appears to be present in measurable amounts after an extended hydrolysis time (90 hours). Only 36 per cent of the total

TABLE III

The nucleosides and nucleoside diphosphates produced by alkali hydrolysis of wheat germ s-ribonucleates

(mole per 100 moles nucleotides)

Nucleosides*		Nucleoside diphosphates	
A	0.995	pAp	0.049
G	0.024	pGp	1.160
C	0.222	pCp	0.013
U	0.050	pUp	0.074
	1.291		1.296

---

[*] Adenine and 2-amino, 6-hydroxy, 5-methyl formamido, 4-phosphoribosyl amino pyrimidine were present in the nucleoside fraction and each accounted for about 0.02 mole per 100 moles of nucleotides in the s-ribonucleate hydrolysates.

ultraviolet absorption (260 mμ) of the guanosine band separated by System 1 is actually guanosine.

The diphosphonucleoside fraction which had been eluted with 1 M pyridine formate was evaporated to dryness in a "flash evaporator". The pyridinium salts of the diphosphonucleosides were converted to ammonium salts by dissolution in 1 M ammonium hydroxide and evaporation to dryness in a "flash evaporator". The nucleoside diphosphates were recovered in a small volume of water in the manner which was described earlier for the nucleosides.

In order to obtain a reliable value for cytidine diphosphate, a relatively large amount (30 s.u.) of the diphosphonucleoside fraction was spotted for paper chromatography in 2 dimensions using System 1 in the first dimension and System 2 in the second dimension.

The proportions of nucleosides and nucleoside diphosphates are shown in Table III. The methods provide for 85 per cent recovery of each nucleoside and 90 per cent recovery of each nucleoside diphosphate and the figures shown in Table III have been corrected to quantitative recovery. From these end group data, the chain length of the s-ribonucleates can be calculated as the reciprocal of the sum of the number of moles of nucleosides (or diphosphonucleosides) per 100 moles of total nucleotides in the alkali hydrolysates, and can be seen to be about $\frac{100}{1.29}$ = 77 nucleotide residues.

(v) The alkali-stable dinucleotides

1. De-salting and recovery from column effluents

(a) Ammonium carbonate de-salting

The effluent, or a fraction thereof, containing approximately 100 s.u. of the dinucleotides was diluted with 4 volumes of water before passage into a 6 x 2.5 cm DEAE cellulose column (carbonate form). The column was carefully washed with 500 ml of water and the dinucleotides were eluted with 100 ml of 1 *M* ammonium carbonate. The effluent was lyophilized to remove ammonium carbonate and the salt-free residue was dissolved in water. Efforts to remove ammonium carbonate by evaporation at 30°-40° in a "flash evaporator" were unsuccessful and therefore an alternative method using pyridine formate as the volatile salt was developed.

(b) Pyridine formate de-salting

The effluent, or a fraction thereof, containing approximately 100 s.u. of the dinucleotides was diluted with 4 volumes of water and passed into a 6 x 2.5 cm DEAE cellulose column (formate form). The column was washed with water (approximately 1000 ml) and the dinucleotides were eluted with 100 ml of 1 *M* pyridine formate, pH 4.5. The effluent was evaporated to dryness in a "flash evaporator" at 30°-40°C. The pyridinium salts were converted to ammonium salts in the manner previously described for the nucleoside diphosphates and the dry residue was dissolved in water and concentrated in an evaporating tube.

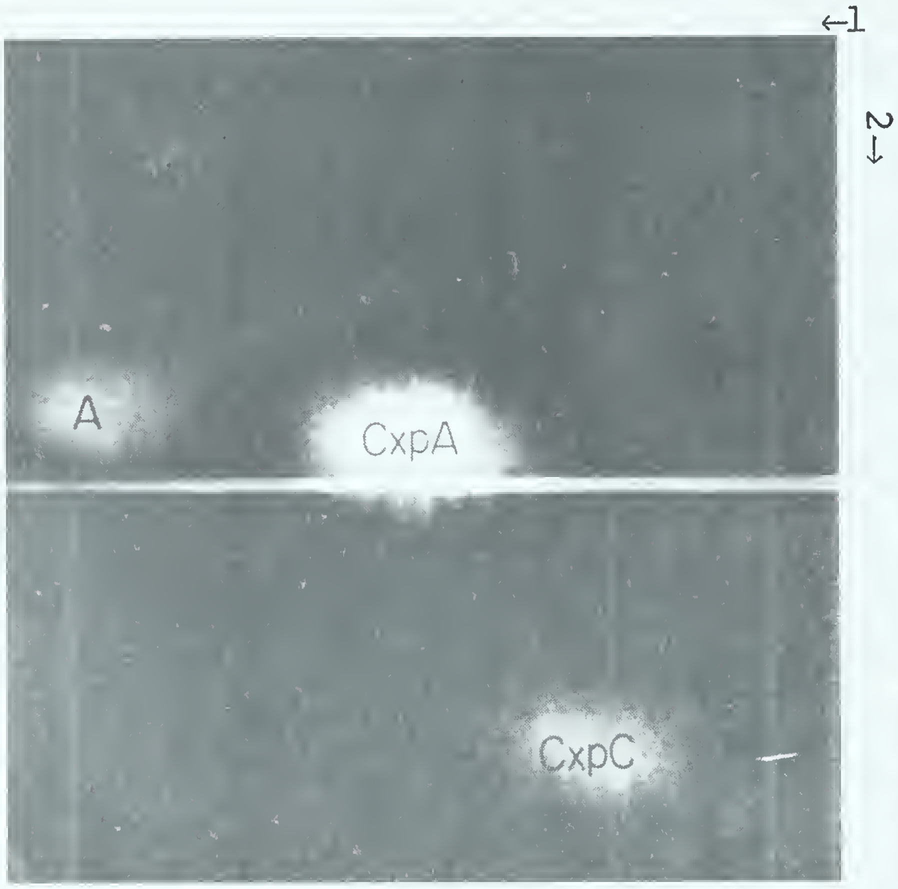
Identical aliquots of the dinucleotides were desalted by

each of the two methods and the dinucleotides recovered were dephosphorylated by treatment with _E. coli_ phosphomonoesterase at 37° for 90 minutes. The dinucleoside phosphates were resolved by paper chromatography. Comparison of the proportions of the dinucleoside phosphates recovered by the two methods showed no significant differences in the relative proportions of the dinucleoside phosphates. The overall recoveries of the dinucleotides by both methods are similar, but the pyridine formate desalting method is preferred because it is more convenient.

2. Separation of individual dinucleotides

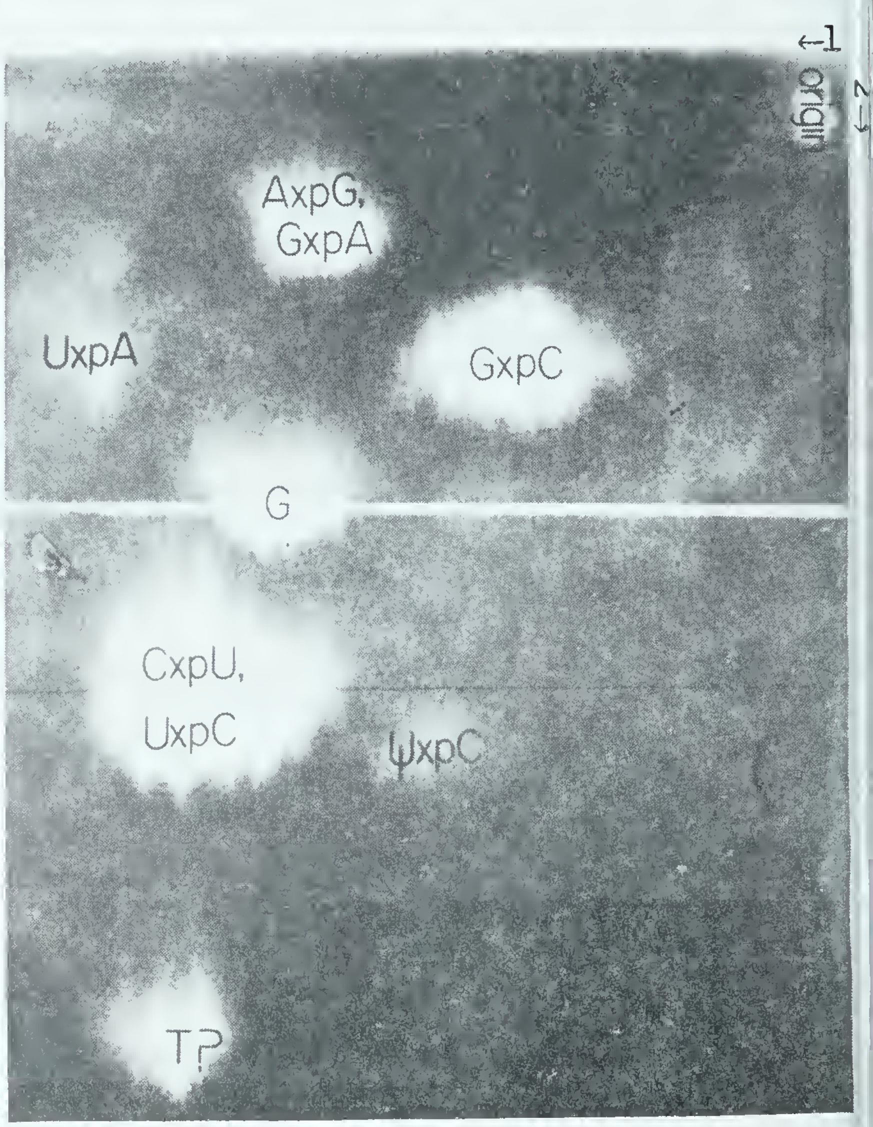
An aliquot of the large scale preparation of alkali-stable dinucleotides (798 s.u.) was passed into a 25 x 1 cm column of DEAE cellulose in the formate form. The column was washed with one volume of water and elution of the dinucleotides was begun with 1 _M_ formic acid, pH 2 (Lane and Allen, 1961b). The first peak from the column (fraction 1) contained the dinucleotides AxpAp, CxpCp, CxpAp, AxpCp and was clearly separated from the second peak (fraction 2) which contained AxpGp, AxpUp, GxpAp, GxpCp, CxpGp, CxpUp, UxpAp and UxpCp. The remaining dinucleotides, GxpGp, GxpUp, UxpGp and UxpUp (fraction 3) were eluted with 1 _M_ pyridine formate, pH 4.5. The pH value of fractions 1 and 2 was adjusted to 4.5 by adding 1 _M_ pyridine. The three fractions were evaporated to dryness and the pyridinium salts were converted to ammonium salts in the manner previously described for the diphosphonucleosides. The separation of the dinucleotides into fractions 1, 2 and 3 prior to resolution by

Ultraviolet photographs depicting the two-dimensional paper chromatographic separation of the components of Fractions 1,2 and 3.



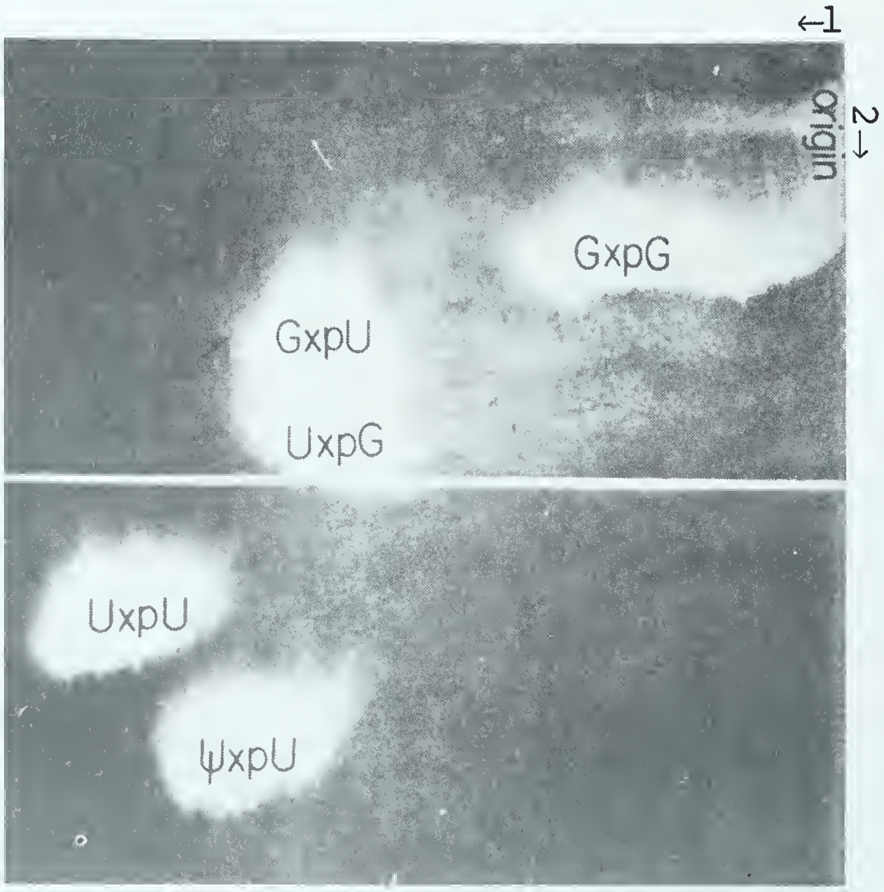
Fraction 1

FIGURE 2



Fraction 2

FIGURE 3



Fraction 3

FIGURE 4

paper chromatography facilitated the detection and measurement of those dinucleotides which comprised less than 2 per cent of the total alkali-stable dinucleotide fraction from alkali hydrolysates of s-ribonucleates.

An aliquot of each dinucleotide fraction (3 μmoles in 200 μl of water) was mixed with 50 μl of 1 M ammonium formate buffer, pH 9.2, and 50 μl of E. coli phosphomonoesterase and then incubated at 37° for 90 minutes. The resulting dinucleoside phosphates were resolved by two-dimensional paper chromatography. System 1 was used for the first dimension, and System 2 for the second dimension. Typical 2-dimensional chromatograms of fractions 1, 2 and 3 are illustrated by the ultraviolet photographs in Figures 2, 3, 4. The compounds were eluted in water or in 0.1 N hydrochloric acid (for quantitative analysis) and were spectrophotometrically characterized between 230 mμ and 340 mμ using the Bausch and Lomb recording spectrophotometer. When two isomers were not resolved by 2-dimensional paper chromatography, the relative amounts of each were determined from the proportions of the hydrolysis products which were formed by phosphodiesterase hydrolysis as described in section 3(b) of this part of the thesis.

3. Characterization of individual dinucleotides

(a) Spectrophotometric characterization

Spectral comparison with an authentic series of alkali-labile dinucleotides (Lane and Allen, 1961b) at pH values 2, 7 and 12 permitted a preliminary assignment to be made of the

identity of the dinucleoside phosphates.

(b) Enzymic degradation

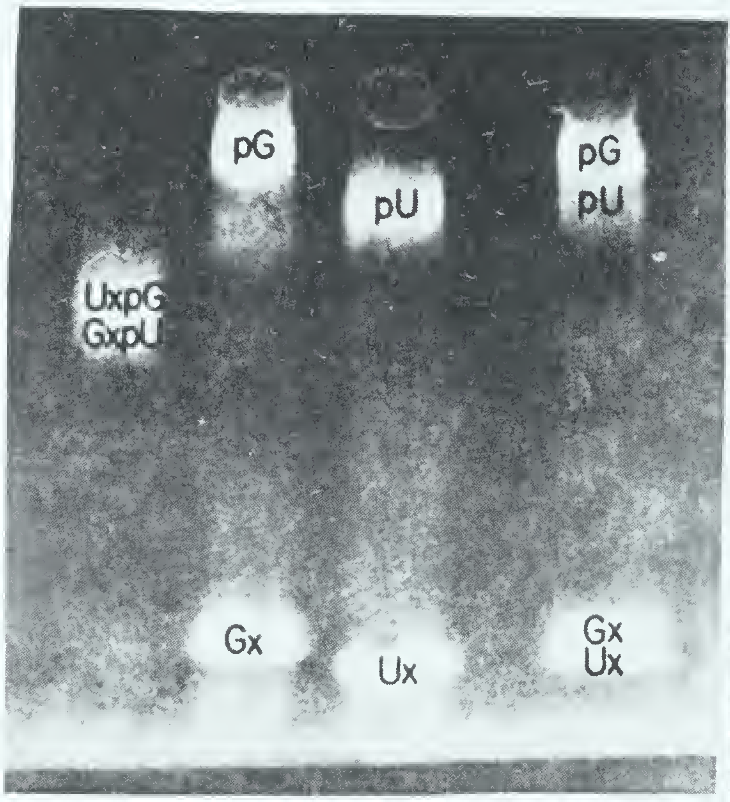
Each dinucleoside phosphate from two-dimensional paper chromatograms was eluted in water and desalted on a charcoal column for characterization by enzymic degradation to the component 2'-0-methyl nucleoside and normal 5'-nucleotide.

$$\mathrm{NxpN'} \longrightarrow \mathrm{Nx} + \mathrm{pN'}$$

Approximately 2 s.u. of each dinucleoside phosphate in 200 μl of water, was mixed with 50 μl of 1 M ammonium formate buffer, pH 9.2, and 200 μl of snake venom phosphodiesterase and the digest was incubated for 24 hours at 37°. The hydrolysis products were resolved by paper chromatography in System 3 and were characterized spectrophotometrically. The amounts of 2'-0-methyl nucleoside and 5'-nucleotide were equimolar in all cases.

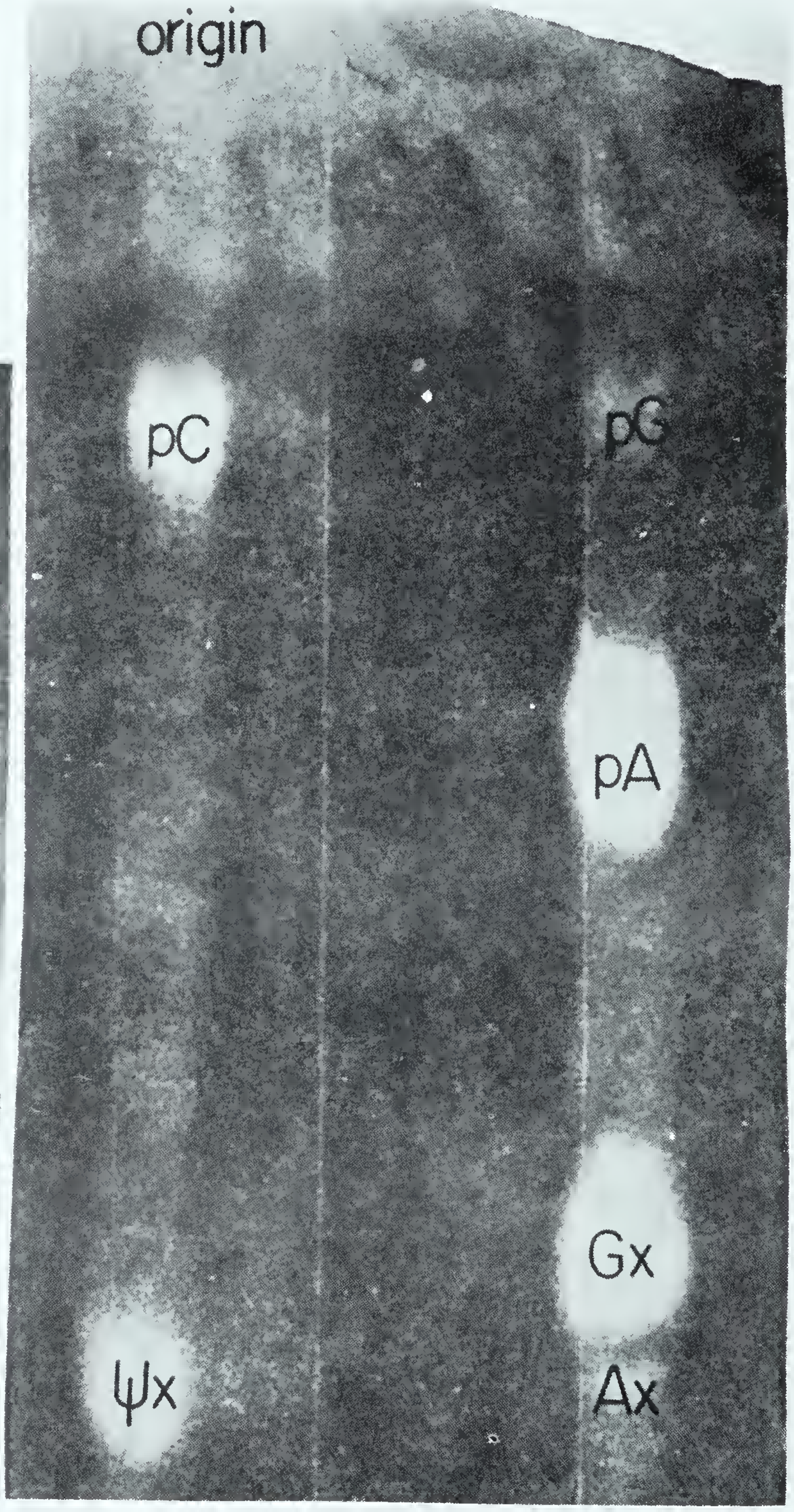
Chromatography in System 3 provides partial characterization of the products on the basis of chromatographic mobilities since the 2'-0-methyl nucleosides do not complex with the borate in the developing solvent and move near the solvent front whereas the 5'-nucleotides complex with the borate and move only a short distance from the origin. Supplementary chromatographic characterization of the compounds using System 1 for separation of the products was obtained in many cases. Thus, when isomeric pairs of dinucleoside phosphates such as AxpG and GxpA, appeared in the same spot after two-dimensional

Ultraviolet photographs of one-dimensional paper chromatograms showing the resolution of the products formed by phosphodiesterase hydrolysis of various dinucleoside phosphates.



System 3

FIGURE 5



System 1

FIGURE 6

chromatography, the four products formed by phosphodiesterase hydrolysis (Ax, Gx, pA, pG) did not resolve in System 3, while satisfactory resolution was possible in System 1. The hydrolysis products formed by phosphodiesterase treatment of the unresolved isomers UxpC and CxpU were also separated by one-dimensional paper chromatography in System 1. The remaining pair of isomers which did not separate adequately in two-dimensional paper chromatograms were GxpU and UxpG, but in this instance the products from phosphodiesterase treatment were nearly resolved in system 3 and completely resolved by one-dimensional chromatography using system 1. Ultraviolet photographs of chromatograms in Figures 5 and 6 illustrate the separation of the hydrolysis products from several dinucleoside phosphates using chromatographic systems 3 and 1.

(c) Periodate degradation

A portion of the dinucleoside phosphate fraction was characterized by periodate oxidation followed by alkaline decomposition at pH 8 and 60° (Neu and Heppel, 1964). The products were the component 2'-0-methyl nucleoside 3'-phosphates and the normal bases.

$$\mathrm{NxpN'} \xrightarrow{\mathrm{IO_4^-}} \mathrm{Nxp} + \mathrm{n'}$$

The dinucleoside phosphate fraction (15 s.u. per 100 μl) was treated with 100 μl of 0.5 M sodium metaperiodate and 100 μl of 1 M lysine, pH 8, for 2.5 hours at 60°. After the initial incubation, 100 μl of 1 M glycol was added and the resulting

TABLE V

Dinucleotide proportions of a typical wheat germ s-ribonucleate preparation

Dinucleotide	Actual recoveries (mole/100 mole) nucleotides)	%M Quantitative recoveries (mole/100 mole nucleotides)	%D (mole/100 mole dinucleotides)	%M Calculated from %D (mole/100 mole nucleotides)
AxpAp	0.0032	0.0042	0.3	0.0042
AxpGp	0.0014	0.0018	0.1	0.0014
AxpCp	0.00	0.00	0.0	0.00
AxpUp	0.00	0.00	0.0	0.00
GxpAp	0.032	0.042	3.1	0.043
GxpGp	0.192	0.249	18.3	0.254
GxpCp	0.082	0.107	7.8	0.108
GxpUp	0.051	0.066	4.9	0.068
CxpAp	0.048	0.062	4.6	0.064
CxpGp	0.00	0.00	0.0	0.00
CxpCp	0.066	0.086	6.3	0.088
CxpUp	0.209	0.272	19.9	0.277
UxpAp	0.007	0.009	0.6	0.008
UxpGp	0.080	0.104	7.6	0.106
UxpCp	0.099	0.129	9.4	0.131
UxpUp	0.091	0.118	8.7	0.121
ψxpCp	0.022	0.029	2.1	0.029
ψxpUp	0.065	0.085	6.2	0.086
Total	1.0486	1.364	100.0	1.39

TABLE IV

Comparison of the quantities of Nxp and pNx recovered from the same preparation of wheat germ s-ribonucleates

(mole/100 moles of nucleotides)

	Ax	Gx	Cx*	Ux	ψx**	Total***
3'-phosphates	0.03	0.44	0.21	0.35	0.09	1.12
5'-phosphates	0.02	0.42	0.38	0.28	0.14	1.24

---

*Much Cxp was lost when the DEAE cellulose column was eluted with the 0.07 M TRIS formate eluent used to remove iodate from the column.

**The ψxp had the R_f value expected for this compound but did not give a satisfactory spectrum in alkaline solution. The pψx had the expected R_f value but appeared to be contaminated with other material since the bathochromic shift in alkali was not as great as expected.

***The pNx values quoted are the averages of two determinations. The values of both the Nxp and the pNx were not corrected to quantitative recovery.

solution was allowed to stand at room temperature for 20 minutes before dilution to 50 ml. The diluted solution was applied to a 3.8 x 2.5 cm DEAE cellulose column (formate form) for separation of the bases from the 2'-0-methyl nucleoside 3'-phosphates. The column was washed with water until the optical density of the effluent was zero. The effluent from the charging and washing solutions, which contained the bases, was desalted on a charcoal column as described earlier for the nucleosides in Part III (iv) of the thesis. The column was washed with 0.07 M TRIS-formate buffer, pH 7.8, to remove the iodate. The TRIS-formate was removed by a thorough washing of the column with water, and the 2'-0-methyl nucleoside 3'-phosphates were eluted with pyridine formate and concentrated into a small volume. Both fractions were separated by chromatography in system 1 for 15 hours. The proportions found for the Nxp derivatives are compared in Table IV to those values obtained for the pNx derivatives (cf. Part IV, ii). The fraction containing the bases was unusual as no compound with the spectrophotometric properties of uracil was obtained. The yields of the other bases were low, and two unusual bands with R_f values greater than uracil but with dissimilar spectrophotometric properties were detected.

4. Results of the quantitative analysis

The actual recovered amounts of individual dinucleotides, expressed as mole percentages, are shown in the first column of Table V. The molar quantity of the nucleotides in

the alkali hydrolysate from which the dinucleotides were isolated was calculated by dividing the total absorbance (260 mμ) of the neutralized hydrolysate by the mean molar extinction coefficient of the nucleotides at pH 7. The mean molar extinction coefficient of the nucleotides, based on the analytical composition of the s-ribonucleates was:

$$\frac{(15\times10^3\times23.1)+(11.4\times10^3\times30.6)+(7.5\times10^3\times27.6)+(10.0\times10^3\times18.7)}{100}$$

$$= 11 \times 10^3$$

The molar quantity of each dinucleotide was obtained by dividing the total absorbance (260 mμ) of the compound recovered from two-dimensional chromatograms by the molar extinction coefficient of the dinucleotide. The molar extinction coefficient of each dinucleotide was calculated as the sum of the molar extinction coefficients of the constituent nucleosides.

The actual recovered amount of each dinucleotide, shown in column 2 of Table V, can be corrected to give the true amount of each dinucleotide as a mole percentage of the total constituent nucleotides of the s-ribonucleates (%M). The per cent recoveries of the dinucleotides observed during the various steps in their isolation agree with known recoveries of other nucleate derivatives during the same procedures, and it is therefore assumed they are true recoveries of dinucleotide material. Thus the values of %M in the third column of

Table V were obtained by multiplying the actual recovered amount of each dinucleotide by $\frac{100}{95}$ (the reciprocal of the per cent recovery during desalting), $\frac{100}{95}$ (the reciprocal of the per cent recovery during the second column fractionation), and $\frac{100}{85}$ (the reciprocal of the per cent recovery from paper chromatograms). The correction factor to convert the actual recovered amounts to quantitative values is therefore:

$$\left(\frac{100}{95}\right)\left(\frac{100}{95}\right)\left(\frac{100}{85}\right) = 1.30$$

In order to compare the relative amounts of the various dinucleotide sequences in the s-ribonucleates, it is useful to express the values for individual dinucleotides as mole percentages of the total dinucleotides (%D). Since there is no destruction or non-uniform loss of dinucleotides during isolation, the comparisons are valid for any given preparation of s-ribonucleates and are independent of the recoveries of the dinucleotides. The values for %D in the fourth column of Table V are calculated by dividing the actual recovered amounts of each dinucleotide (column 2 of Table V) by the sum of the recovered amounts of all the dinucleotides (the sum of the figures in column 2 of Table V).

The values for the quantitative recovery (%M) of the dinucleotides can be checked by converting %D to %M. Assuming all the s.u. in the dinucleotide fraction from the initial column separation are contributed by dinucleotides, then the total amount of all dinucleotides expressed as %M would be:

$$100 \times \frac{\dfrac{\text{(s.u. in the dinucleotide fraction)}}{\text{(mean molar extinction coefficient of the dinucleotides)}}}{\dfrac{\text{(s.u. in the hydrolysate)}}{\text{(mean molar extinction coefficient of the nucleotides)}}}$$

The mean molar extinction coefficient (260 mμ) of the dinucleotides in the hydrolysates of s-ribonucleates at pH 7.8 is calculated using the observed recoveries and the molar extinction coefficients of the dinucleotides.[*] The total amount of dinucleotides, expressed as %M would then be:

$$100 \times \frac{\dfrac{900}{19.6}}{\dfrac{36400}{11}} = 1.39$$

The amount of each dinucleotide expressed as %M was calculated by multiplying the %D of each dinucleotide (expressed as a decimal fraction) by 1.39. The values obtained by this calculation are shown in column 5 of Table V. The similarity of the values for %M in columns 3 and 5 lends strong support to the assumptions that the presumed recoveries are accurate and that all the s.u. in the dinucleotide fraction are attributable to dinucleotides.

(vi) Reproducibility of the analytical data

Earlier analyses were performed in the course of refining the experimental techniques and the accumulated data show

---

[*] $$10^3 \times \frac{(0.003\times29.8)+(0.001\times26.3)+(0.032\times26.3)+(0.192\times23.4)+}{1.049}$$
$$\frac{(0.082\times19.3)+(0.051\times21.7)+(0.048\times19.5)+(0.066\times15.1)+(0.209\times17.5)}{1.049}$$
$$\frac{+(0.007\times24.9)+(0.080\times21.7)+(0.099\times17.5)+(0.091\times19.9)+(0.022\times14.9)}{1.049}$$
$$\frac{+(0.065\times17.4)}{1.049} = 19.6\times10^3$$

TABLE VII

Dinucleotide analyses of alkali-hydrolysates of several wheat germ s-ribonucleate preparations

	(Expressed as %D; mole/100 moles of dinucleotides)					
Dinucleotide sequence	1	2	3	4	5	Mean values
AxpAp	1.4	3.0	0.3	0.3	0.9	1.2
AxpGp	0.4	0.9	0.4	0.1	0.3	0.4
AxpCp	0.0	0.0	0.0	0.0	0.0	0.0
AxpUp	0.0	0.0	0.0	0.0	0.0	0.0
GxpAp	3.5	7.5	2.9	3.0	7.8	4.9
GxpGp	19.5	13.6	12.1	18.0	16.2	15.9
GxpCp	7.7	10.9	5.1	7.9	10.5	8.4
GxpUp	5.4	4.4	4.2	4.0	3.9	4.4
CxpAp	5.0	7.1	6.8	4.8	6.8	6.1
CxpGp	0.0	0.0	0.0	0.0	0.0	0.0
CxpCp	6.5	6.0	10.3	6.7	7.2	7.4
CxpUp	18.2	22.1	24.3	19.2	17.1	20.2
UxpAp	1.7	0.2	0.0	0.6	3.4	1.5
UxpGp	7.5	5.6	5.8	8.3	6.1	6.7
UxpCp	7.6	9.3	13.9	10.3	10.7	10.4
UxpUp	9.2	8.3	7.4	8.6	8.5	8.4
ψxpCp	2.3	0.0	2.0	2.2	0.0	2.2
ψxpUp	5.1	1.8	4.4	6.1	2.5	4.0

TABLE VI

Nucleoside and diphosphonucleoside analyses of alkali hydrolysates of several wheat germ s-ribonucleate preparations

	Nucleosides						Diphosphonucleosides					
	A	G	C	U	ΣN	Chain length	pAp	pGp	pCp	pUp	ΣpNp	Chain length
1	1.013	0.009	0.145	0.039	1.206	83	0.085	1.035	0.0	0.148	1.268	79
2	0.920	0.011	0.234	0.042	1.207	83	0.067	1.096	0.0	0.0	1.163	86
3	0.980	0.028	0.214	0.058	1.280	78	0.035	1.248	0.019	0.088	1.390	72
4	0.995	0.024	0.222	0.050	1.291	77	0.049	1.160	0.013	0.074	1.296	77
5	1.042	0.014	0.232	0.039	1.327	76	0.023	1.100	0.042	0.075	1.240	81

that there is little variation among the nucleoside, diphosphonucleoside and dinucleotide proportions of several independent preparations of s-ribonucleates. The earlier analyses of the dinucleotides were performed without a preliminary column separation into fractions 1, 2 and 3. It might have been expected that the measured proportions of the dinucleotide sequences in the s-ribonucleates would vary from one preparation to another since there is an average of only one dinucleotide sequence per chain. Loss of any given type of s-ribonucleate chain during isolation could result in the specific loss of one type of sequence. However, in practice, there have been no striking differences in the relative or absolute amounts of the dinucleotides from one preparation to another. Analyses for the relative amounts of isomeric pairs such as UxpCp and CxpUp were not done on all preparations, but where analyses were performed they agreed well with the results of section (v).

The data for several nucleoside and diphosphonucleoside analyses are given in Table VI. The data presented in Table VII show the analyses of the dinucleotide fractions from several preparations.

IV. ANALYSIS OF SNAKE VENOM PHOSPHODIESTERASE HYDROLYSATES OF WHEAT GERM s-RIBONUCLEATES

(i) Hydrolysis of s-ribonucleates by purified snake venom phosphodiesterase

1. Conditions for hydrolysis and the nature of the products

The s-ribonucleates (50 mg) were dissolved in 5 ml of water and the resulting solution was mixed with 2.5 ml of 1 *M* ammonium formate buffer, pH 9.2, and 2.5 ml of purified phosphodiesterase devoid of phosphomonoesterase activity. The hydrolysate was incubated at 37° for 24 hours. The phosphodiesterase hydrolyzed about 15 mg of the ribonucleates to 5'-nucleotides during the first hour of digestion under these conditions, and digestion was complete after 24 hours.

The principal products of hydrolysis are the major 5'-nucleotides (pN) together with smaller amounts of the 5'-nucleotides which have methyl substituents on the heterocycle (pN^x) and on the 2'-position of ribose (pNx).

2. Analysis of the major 5'-nucleotides and the minor 5'-nucleotides which have methyl substituents on the heterocycle

The hydrolysis products produced by purified snake venom phosphodiesterase hydrolysis of the s-ribonucleates were separated by two-dimensional paper chromatography, using System 1

TABLE VIII

The analytical composition of phosphodiesterase hydrolysates of wheat germ s-ribonucleates

Component	Molar extinction coefficient at 260 mμ (x 10^{-3})	Mole per cent of to 5'-nucleotides	
pA	14.2	19.8	(23.2)*
pAx	14.2	0.023	(0.60)
1MepA	14.2	0.19	
N^{6}MepA	17.7	0.78	
A	14.3	0.039	
pG	11.8	29.3	(30.1)
pGx	11.8	0.41	(0.44)
1MepG	12.2	0.78	
7MepG	11.8	0.19	
N^{2}MepG	13.1	0.29	
N^2,N^{2}diMepG	14.5	0.58	
G	11.8	0.058	
pC	6.2	26.3	(24.8)
pCx	6.2	0.37	(0.42)
5MepC	3.2	1.46	
C	6.4	0.039	
pU	10.0	15.7	(18.1)
pUx	10.0	0.29	(0.51)
5MepU	8.7	0.68	
U	10.0	0.029	
pψ	7.5	2.8	(1.8)
pψx	7.5	0.16	

[*] Values in parentheses are the corresponding figures for 18S + 28S ribonucleates.

Ultraviolet photograph of a two-dimensional paper chromatogram showing the separation of the products formed by phosphodiesterase hydrolysis of wheat germ s-ribonucleates.

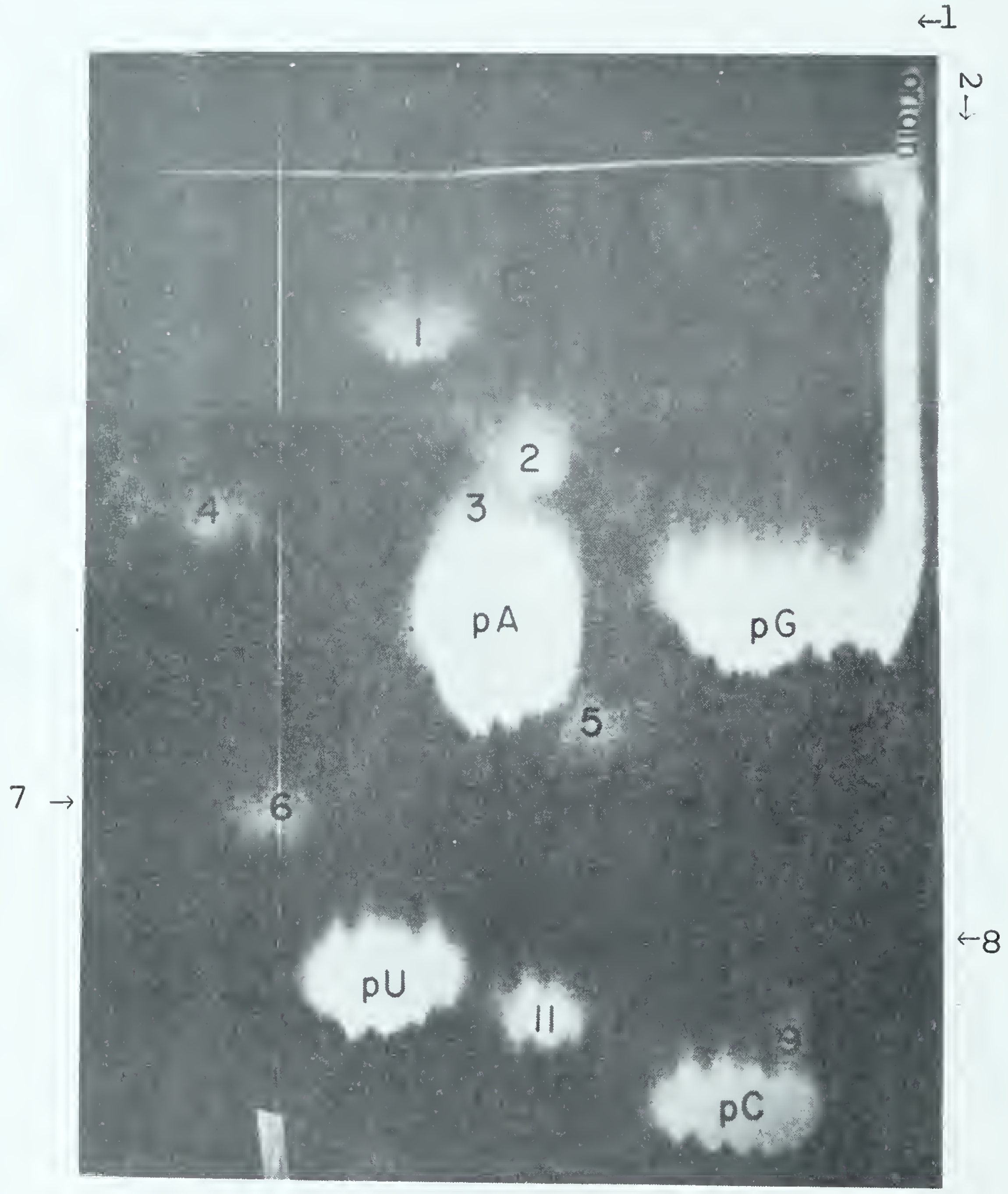


FIGURE 7

1. N^2-di-Me-pG
2. 1-Me-pG
3. N^2-Me-pG
4. N^6-Me-pA
5. pI
6. pT
7. pUx
8. 7-Me-pG
9. 1-Me-pA
10. 5-Me-pC
11. pΨ

in the first dimension and System 2 in the second dimension. Approximately 60 s.u. of the nucleotide material was spotted for chromatographic separation and the separated compounds were eluted in 0.1 M hydrochloric acid. An ultraviolet photograph of a typical chromatogram is shown in Figure 7.

The major components were eluted in 25 - 50 ml of 0.1 M hydrochloric acid solution and the minor components were eluted in 2 - 4 ml of 0.1 M hydrochloric acid solution. The molar quantities of the components were estimated from their absorbance at 260 mμ using the molar extinction coefficients listed in Table VIII. The mean results of several analyses are shown in Table VIII and the values for minor components seldom showed greater than ± 10 per cent variation for independent preparations of the s-ribonucleates. In most instances there are five or six determinations contributing to the mean values of Table VIII. It is notable that the values for the major components showed less than ± 2 per cent variation from one preparation to another.

The figures quoted for the four nucleosides and 1-MepA in phosphodiesterase hydrolysates were obtained by passage of the hydrolysate through a DEAE cellulose column and analysis of the material which did not adsorb to DEAE cellulose.

The molar extinction coefficients of pN^x compounds were taken to be the same as the values for the nucleosides which were quoted by Dunn *et al.* (Littlefield and Dunn, 1958; Smith and Dunn, 1959b), and the molar extinction coefficients of pNx

compounds were presumed to be identical with those of the corresponding pN compounds. The molar extinction coefficient of 5-MepC was presumed to be the same as the value for the corresponding deoxyribonucleotide and the extinction coefficients of 1-MepA and 7-MepG were presumed to be the same as the values for pA and pG, respectively.

3. The rate of hydrolysis of different internucleoside linkages by snake venom phosphodiesterase

Nihei and Cantoni (1963) used purified snake venom phosphodiesterase to hydrolyze s-ribonucleates by the step-wise removal of 5'-mononucleotides from the 5'-linked terminus. It was assumed that the enzyme had no substrate preference, and would attack different internucleoside linkages at the same rate. Comparison of the rates of phosphodiesterase hydrolysis of two different substrates shows this assumption to be questionable.

The alkali-labile dinucleotide, ApAp, and the alkali-stable dinucleotide, AxpAp, were isolated from 18S + 28S ribonucleates and hydrolyzed with phosphodiesterase after dephosphorylation to ApA and AxpA, respectively.

A comparison of the hydrolysis rates of 18S + 28S ribonucleates, ApA and AxpA by snake venom phosphodiesterase is shown in Figure 8. Ribonucleate digestion was followed by measuring the release of 5'-nucleotides as products soluble in trichloroacetic acid solution while dinucleoside phosphate hydrolysis was measured by paper chromatographic separation of

The rates of hydrolysis of ApA, AxpA and ribonucleates by purified snake venom phosphodiesterase

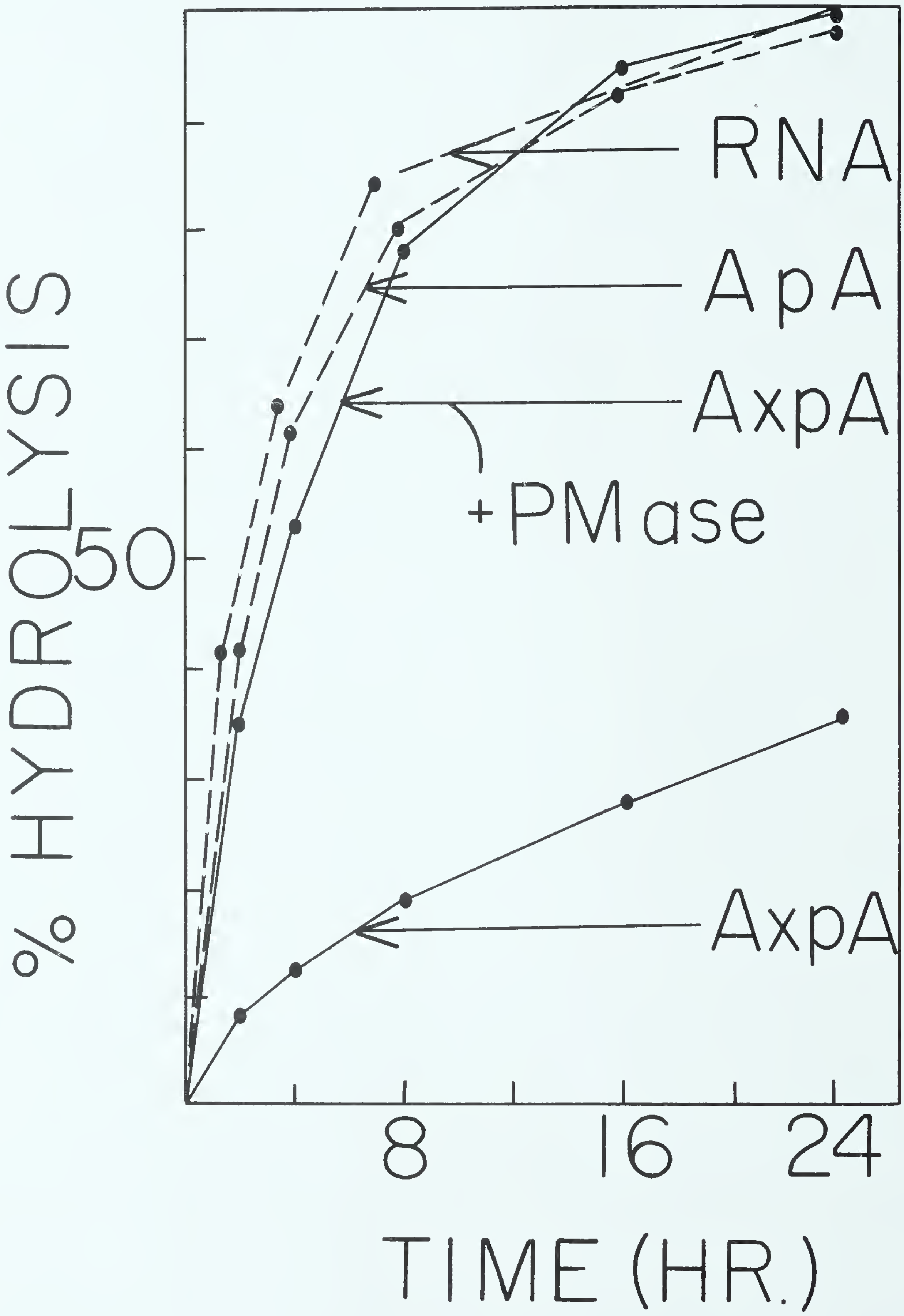


FIGURE 8

substrate and products using System 2. The digestions were performed in 0.25 M ammonium formate buffer, pH 9.2, with a substrate concentration equivalent to about 11 μmoles of constituent nucleotides per ml of digest. The enzyme concentration in dinucleoside phosphate digests was 80 per cent of the concentration used for ribonucleate digestion. Column purified *E. coli* phosphomonoesterase (50 μl per ml of digest) was added to one of the AxpA digests as indicated in Figure 8.

It is evident that the rates of hydrolysis of internucleoside linkages in ribonucleates and in ApA are similar but that the hydrolysis of AxpA proceeds more slowly. The rate of hydrolysis of AxpA is very similar to that for ApA when phosphomonoesterase is present and this indicates that AxpA hydrolysis by phosphodiesterase is severely inhibited by the 5-nucleotide, pA. The extent of pN-inhibition of the hydrolysis of NxpN linkages in ribonucleate chains may not be as great as found for the dinucleoside phosphate but the result with the dinucleoside phosphate seems to warrant caution in the interpretation of results such as those obtained by Nihei and Cantoni (1963). Any attempt to assess the spatial distribution of Nx and N^x residues in ribonucleate chains by hydrolysis with snake venom phosphodiesterase would need to be predicated on a more extensive investigation of the type outlined above.

(ii) Hydrolysis of s-ribonucleates by whole snake venom

1. Conditions for hydrolysis and the nature of the products

The s-ribonucleates (50 mg) were dissolved in 5 ml of water and the resulting solution was mixed with 2.5 ml of 1 *M* ammonium formate buffer, pH 9.2, and 2.5 ml of a 0.25 per cent aqueous solution of Russell Viper Venom. The hydrolysate was incubated at 37° for 24 hours before neutralization with 1 *M* formic acid.

The principal products formed are the nucleosides adenosine, guanosine, cytidine, uridine and pseudouridine (N), together with small amounts of the nucleosides which have methyl substituents on the heterocycle (N^x). The 2'-O-methyl nucleoside 5'-phosphates are not dephosphorylated by the 5'-nucleotidase in whole snake venom (Honjo *et al.*, 1964) and can be easily separated from the bulk of the hydrolysis products which have been dephosphorylated.

2. Analysis of the minor 5'-nucleotides having 2'-O-methyl substituents

The whole snake venom hydrolysates of s-ribonucleates were neutralized after dilution to 100 ml with water. The diluted solution was applied to a 6 x 2.5 cm DEAE cellulose column in the formate form, which had been pre-equilibrated with 0.025 *M* TRIS-formate buffer, pH 7.8. After the charging solution had passed through the column, elution was continued with 0.032 *M* TRIS-formate buffer, pH 7.8, until the optical

density (260 mμ) of the effluent was zero. The effluent from charging and washing contained only nucleosides and was discarded.

The 2'-O-methyl nucleoside-5'-phosphates were eluted by an aqueous solution which was 0.085 M with respect to TRIS-formate buffer, pH 7.8 and 7 M with respect to urea. The elution was continued until the optical density (260 mμ) decreased to that of the eluting buffer (approximately 0.030).

The effluent was diluted with four volumes of water and passed into a 6 x 2.5 cm column of DEAE cellulose (formate form). The column was washed thoroughly with water and the pNx compounds were recovered in the same manner as previously described for the diphosphonucleosides (Part III, iv). An aliquot of the pNx fraction (7 s.u.) was subjected to paper chromatography in System 1 and the bands corresponding to pAx, pGx, pCx, pUx and pΨx were eluted and spectrophotometrically characterized. The results of the quantitative analyses for pNx compounds are shown in Tables IV and VIII.

(iii) Summary of data on the nucleotide composition of wheat germ s-ribonucleates

The four principal 5'-nucleotides (pA, pG, pC and pU) account for about 91 mole per cent of the total nucleotides formed by phosphodiesterase hydrolysis of wheat germ s-ribonucleates. The minor component with the unique C-ribosyl

linkage, 5'-pseudouridylate, accounts for 2.8 mole per cent of the total nucleotides. The remaining 5'-nucleotides have a methyl substituent in either the heterocycle (pN^x), or in the sugar (pNx), and they account for about 5 mole per cent and 1.3 mole per cent of the total nucleotides, respectively.

The findings with s-ribonucleates contrast with the results of corresponding analyses of the 18S + 28S ribonucleates from wheat germ, where the principal 5'-nucleotides account for about 96 mole per cent, 5'-pseudouridylate accounts for 1.8 mole per cent and the pNx compounds account for 2 mole per cent of the total nucleotides, while pN^x compounds are not detectable. The s-ribonucleates and 18S + 28S ribonucleates do not show marked differences in their proportions of the major 5'-nucleotides or of 5'-pseudouridylate but do show a most striking difference in the proportions of the pNx compounds. Thus, pAx is barely detectable in the s-ribonucleates, while it is the major pNx component of the 18S + 28S ribonucleates.

The pN^x compounds have not been previously isolated from the s-ribonucleates from any other source and it was therefore necessary to establish their identity. The pN^x compounds were pooled and it was established that they were susceptible to the 5'-nucleotidase of Russell Viper Venom under conditions where the normal 5'-nucleotides were completely converted to nucleosides but 2'- and 3'-nucleotides were unaffected (1 ml of digest contained 1.0 μmole of nucleotide, 3.3 mg of Russell Viper Venom and 150 μmoles ammonium formate buffer, pH 9.2, and was incubated

at 37^o for 2.5 hours). The pN^x compounds were also shown to be periodate-susceptible. This is the most complete demonstration that the nucleate derivatives having methyl substituents in the heterocycle are recoverable as 5'-nucleotides. This establishes that pN^x compounds participate in the customary 3'-5' internucleoside phosphodiester linkages.

The pNx compounds are not susceptible to either the 5'-nucleotidase of snake venom or to periodate oxidation, and the conclusion that they are indeed 5'-nucleotides rests on the work of Honjo and co-workers (1964).

V. SUMMARY

Several preparations of wheat germ s-ribonucleates were characterized by physico-chemical methods and shown to be homogeneous. The intrinsic sedimentation coefficient, $S^{o}_{20,w}$, was 3.4S when measurements were performed with ultraviolet absorption optics at a ribonucleate concentration of 0.0025 per cent in 0.15 M sodium chloride solution. The weight average molecular weight of the s-ribonucleates based on $S^{o}_{20,w}$, $D^{o}_{20,w}$, and $\bar{v}$ was 31,700, and was in favorable agreement with the number average value of 26,565 based on the average chain length of 77 from end group analysis.

Analysis of the end groups (Table III) by alkali hydrolysis of the wheat germ s-ribonucleates agreed qualitatively with published data on the s-ribonucleates from other sources in that the major diphosphonucleoside was guanosine diphosphate and the major nucleoside was adenosine. Small quantities of the other diphosphonucleosides and nucleosides were also detected. The analytical composition of the wheat germ s-ribonucleates (cf. Table VIII) was typical of s-ribonucleates from other sources in that guanylate and cytidylate accounted for 60 mole per cent of the major nucleotides, while adenylate and uridylate accounted for only 40 per cent of the major nucleotides. Since guanylate and cytidylate are the major nucleotide components of the wheat germ s-ribonucleates, it is perhaps significant that the nucleotides with methyl substituents on guanylate and cytidylate predominate

over the methylated derivatives of adenylate and uridylate.

Glitz and Dekker (1963) isolated the nucleoside derivatives with methyl substituents on the heterocycle as nucleoside 2'- and 3'-monophosphates (N^xp) from alkali hydrolysates. The treatment of wheat germ s-ribonucleates by snake venom phosphodiesterase, and the isolation of the methylated derivatives as nucleoside 5'-monophosphates (pN^x) in this investigation has demonstrated that these nucleosides participate, as expected, in the customary 3'-5' phosphodiester linkages of s-ribonucleate chains.

Pseudouridylate was also isolated as nucleoside 2'- and 3'-monophosphates from alkali digests by Glitz and Dekker (1963). The hydrolysis of wheat germ s-ribonucleates by snake venom phosphodiesterase and the isolation of pseudouridylate as the nucleoside 5'-monophosphate in this investigation has demonstrated that this nucleoside also participates in the usual 3'-5' phosphodiester linkages of s-ribonucleate chains.

The treatment of the dinucleoside phosphates from alkali hydrolysates with periodate and subsequent alkaline decomposition of the dialdehyde gave compounds with the properties expected of 2'-0-methyl nucleoside 3'-monophosphates. Isolation of compounds with the properties expected of 2'-0-methyl nucleoside 5'-monophosphates from whole venom hydrolysates of s-ribonucleates has shown that the 2'-0-methyl nucleosides also participate in the customary 3'-5' phosphodiester linkages of s-ribonucleate chains. The 2'-0-methyl pseudouridine 5'-phosphate isolated from wheat germ s-ribonucleates substantiated the isolation and identification of the corresponding nucleoside by Hall (1964).

TABLE IX

The dinucleotide proportions of wheat germ 18S + 28S ribonucleates and s-ribonucleates

Dinucleotide sequence	%D (moles/100 moles dinucleotides)		%M (moles/100 moles nucleotides)	
	18S + 28S ribonucleates	s-ribonucleates	18S + 28S ribonucleates	s-ribonucleates
AxpAp	8.8	0.3	0.145	0.004
AxpGp	11.2	0.1	0.185	0.001
AxpCp	8.1	0.0	0.135	0.00
AxpUp	7.8	0.0	0.130	0.00
GxpAp	3.1	3.1	0.051	0.041
GxpGp	7.8	18.2	0.130	0.249
GxpCp	5.8	7.9	0.096	0.107
GxpUp	3.0	5.0	0.049	0.068
CxpAp	4.8	4.7	0.080	0.064
CxpGp	0.9	0.0	0.015	0.00
CxpCp	5.4	6.3	0.089	0.086
CxpUp	7.0	18.7	0.117	0.253
UxpAp	8.5	0.6	0.140	0.008
UxpGp	6.1	7.8	0.101	0.105
UxpCp	5.8	10.0	0.096	0.135
UxpUp	5.9	8.8	0.098	0.120
ψxpCp	0.0	2.1		0.029
ψxpUp	0.0	6.3		0.086
	100.0	99.9	1.658	1.356

* The values of %M for the 18S + 28S ribonucleates are calculated from the total %D (1.65 mole %) and the %D of the individual dinucleotides.

The 2'-0-methyl pseudouridylate is a component of two alkali-stable dinucleotide sequences, ψxpCp and ψxpUp.

The alkali-stable dinucleotide sequences of s-ribonucleate chains can be quantitatively isolated because of the unique chemical stability of their internucleoside bonds and the intrinsic non-overlapping nature of the sequences. There are no other sequences in s-ribonucleate chains which are amenable to quantitative isolation. Fifteen alkali-stable dinucleotide sequences have been identified in the alkali hydrolysates of wheat germ s-ribonucleates, and they account for 2.6 mole per cent of the total nucleotides. Since the chain length of the ribonucleates is about 77, then, if the alkali-stable dinucleotide sequences are randomly distributed throughout all s-ribonucleate chains, there would be one such sequence per chain. In the 18S + 28S ribonucleates, there are 16 alkali-stable dinucleotide sequences which account for 3.4 mole per cent of the total nucleotides. If the dinucleotides are randomly distributed throughout all 18S + 28S ribonucleate chains, then all 16 of the possible dinucleotide sequences could be present in every chain since the average chain length is 1300.

For comparative purposes, the %D and %M for both the 18S + 28S ribonucleates and the s-ribonucleates from wheat germ are listed in Table IX. Large differences are seen between the relative amounts of dinucleotide sequences from the two sources when the results are expressed as %D. The amounts of the alkali-stable dinucleotide sequences containing 2'-0-methyl adenylate are almost negligible in the s-ribonucleates, while in the 18S +

28S ribonucleates, they account for the largest portion of the total alkali-stable dinucleotide sequences. The relative quantities of GxpGp, CxpUp and UxpCp in the s-ribonucleates are twice as great as the corresponding values for these same sequences in the 18S + 28S ribonucleates. Pseudouridylate is present in the s-ribonucleates as a 2'-0-methyl nucleoside constituent of the alkali-stable dinucleotide sequences but has not been detected as the normal nucleoside constituent of an alkali-stable dinucleotide sequence.

The <u>absolute</u> quantities of the alkali-stable dinucleotide sequences, expressed as %M, tend to show similarities which are less obvious when the <u>relative</u> amounts (%D) of the sequences in the 18S + 28S ribonucleates are compared with the corresponding figures for the s-ribonucleates.

A comparison of the absolute amounts of individual dinucleotide sequences shows substantial similarities between the 18S + 28S ribonucleates and the s-ribonucleates except in the case of UxpAp and the Ax-containing dinucleotides. Although the absolute amounts of GxpGp and CxpUp isolated from s-ribonucleates are greater than the amounts from 18S + 28S ribonucleates, it can be seen that these sequences do, in both sources, contain the largest amount of Gx and Cx, respectively.

Morisawa and Chargaff (1963) stated that they found no 2'-0-methyl adenylate in liver ribonucleates and yeast s-ribonucleates. The quantitative techniques used in the present studies show that 2'-0-methyl adenylate is present in small

quantity in the wheat germ s-ribonucleates. Similar small quantities of 2'-0-methyl adenosine have been found in the s-ribonucleates of E. coli by Hall (1964).

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